

DOI:

PHOTOSYNTHETICA 57 (X): XXX-XXX, 2019

# Effects of salicylic acid on photosynthetic activity and chloroplast morphology under light and prolonged darkness

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## Abstract

Salicylic acid (SA) is a key component of plant defence, which exerts a concentration-dependent effect on photosynthesis under multi-faceted influence of light. Photosynthetic activities and chloroplast morphology were studied in tomato plants after treatment with a sublethal, 0.1 mM, and a cell death-inducing, 1 mM concentrations of SA under normal photoperiod during light phase and after a prolonged dark phase. SA (1 mM) decreased the maximal ( $F_v/F_m$ ) and effective quantum yields of PSII [ $Y_{(II)}$ ] and PSI [ $Y_{(I)}$ ] under both environmental conditions, however, the photoprotective processes were not significantly different between light and dark samples. Decrease in grana height, thylakoid dilation and deformation of lumen were also observed in the light. In contrast to illuminated samples, 0.1 mM SA decreased  $Y_{(II)}$  and  $Y_{(I)}$  after dark incubation, but nonphotochemical energy dissipation and cyclic electron flow increased, suggesting that the photoprotective mechanisms could be activated in plants exposed to prolonged darkness.

*Additional key words:* carotenoid; nonphotochemical quenching; photosystem II and I; soluble sugar; starch.

## Introduction

Environmental factors exert an influence on growth and development as well as on stress responses of plants. In particular, light can alter plant developmental or defence responses and can induce new signalling and regulation pathways mediated by various hormones (Roberts and Paul 2006, Ballaré 2014), since the concentration and physiological effects of these compounds are modulated by day/night cycles and light quality (Griebel and Zeier 2008, Kazan and Manners 2011, Cerrudo *et al.* 2012, de Wit *et al.* 2013, Kegge *et al.* 2013). The changes in plant primary and secondary metabolism in the dark can alter the availability of current photosynthates, which determine the ability of plants to synthesize defence compounds during the dark period (Kangasjärvi *et al.* 2012, López-Goldar *et al.* 2016, Panchal and Melotto 2017). Concomitantly, many pathogens and insects can perceive these changes adapting their behaviour accordingly (Suárez-Vidal *et al.* 2017). Thus, the clarification of plant metabolism

and stress responses regulated by defence hormones is particularly important under light deprivation.

Salicylic acid (SA) is one of the key components of plant defence signalling networks in local and systemic acquired resistance against many pathogens (Vos *et al.* 2013) but it induces acclimation to various abiotic stresses, too (Horváth *et al.* 2007, Hayat *et al.* 2010, Khan *et al.* 2015). As a stress-related signalling compound, SA plays an indirect or direct role in various physiological processes, including photosynthesis (Mateo *et al.* 2006, Janda *et al.* 2014). Based on several investigations, high concentration (1–5 mM) of exogenously applied SA induces stomatal closure and may therefore be expected to affect the rate of photosynthesis (Poór *et al.* 2011, Poór and Tari 2012). High concentration of SA caused a reduction in the net photosynthetic rate ( $P_N$ ) and decreased the activity and protein content of Rubisco in barley plants (Pancheva *et al.* 1996, Pancheva and Popova 1998). Moreover, chlorophyll (Chl) and carotenoid (Car) contents were also declined after exposure to high concentrations of SA (Fariduddin *et*

*Received* 13 March 2018, *accepted* 22 October 2018.

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**Abbreviations:** Car – carotenoids; CEF – cyclic electron flow; Chl – chlorophyll;  $F_0$  – minimal fluorescence yield in dark-adapted state; FM – fresh mass;  $F_v/F_m$  – maximum quantum yield of PSII; HR – hypersensitive response; LEF – linear electron flow;  $P_N$  – net photosynthetic rate;  $q_L$  – estimates the fraction of open PSII centres;  $q_P$  – photochemical quenching; RC – reaction centres; ROS – reactive oxygen species; SA – salicylic acid; TEM – transmission electron micrograph;  $Y_{(I)}$  – effective quantum yield of PSI;  $Y_{(II)}$  – effective quantum yield of PSII;  $Y_{(CEF)}$  – yield of cyclic electron flow around PSI;  $Y_{(NA)}$  – the quantum yield of nonphotochemical energy dissipation in PSI due to acceptor side limitation;  $Y_{(ND)}$  – quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation;  $Y_{(NO)}$  – quantum yield of nonregulated nonphotochemical energy dissipation;  $Y_{(NPQ)}$  – quantum yield of regulated non-photochemical energy dissipation.

**Acknowledgements:** We thank Bécs Attiláné for her excellent technical assistance. This work was supported by grant from the Hungarian National Scientific Research Foundation (OTKA PD112855). The authors declare that they have no conflict of interest.

*al.* 2003, Moharekar *et al.* 2003).

Increasing SA concentrations resulted in a significant decrease in the maximum ( $F_v/F_m$ ) and effective quantum yields ( $\Phi_{PSII}$ ) of PSII and also in the photochemical quenching coefficient ( $q_p$ ), and an increase in the nonphotochemical quenching (NPQ) in tomato leaves and guard cells (Poór and Tari 2012). In another investigation, it was observed that PSII electron transport was more sensitive to high SA than PSI in SA-infiltrated tobacco leaves (Janda *et al.* 2012). Nevertheless, the effects of SA on PSI received less attention.

A treatment of barley by 1 mM SA not only decreased the PSII activity but also induced alterations in leaf anatomy and reduced the width of the adaxial and abaxial epidermis. In parallel with decreasing photosynthetic pigment contents, the chloroplast ultrastructure was also affected by high concentrations of SA. Swelling of grana thylakoids, coagulation of the stroma, and increase in chloroplast volume were observed in these samples (Uzunova and Popova 2000). On the other hand, many scientific reports indicated that SA in micromolar ( $\mu\text{M}$ ) concentration plays a pivotal role in the protection of the photosynthetic apparatus of plants that are exposed to several environmental stresses (Rivas-San Vicente and Plasencia 2011), such as cold (Janda *et al.* 2007), heat (Hayat *et al.* 2009), drought (Habibi 2012), salt (Poór *et al.* 2011) or heavy metal stress (Krantev *et al.* 2008). However, it can be concluded that the effect of SA depends on the applied concentration, on the duration and the mode of the application, on plant species, on developmental stage or on environmental conditions, such as the presence or quality of light (Hayat *et al.* 2010, Rivas-San Vicente and Plasencia 2011). Although, it was observed that accumulation of SA and the initiation of SA-mediated signalling pathways after pathogen infection are also light-dependent (Chandra-Shekara *et al.* 2006, Griebel and Zeier 2008, Goodspeed *et al.* 2012, Karpiński *et al.* 2013, Poór *et al.* 2017), the effects of SA on the photosynthetic apparatus and on the efficiency of photosynthesis after a prolonged dark period remained mostly unclear. At the same time, the SA-mediated changes in photosynthetic performance can markedly influence the defence responses of plants after pathogen infection in the dark (Cheng *et al.* 2016).

In this article, comparative analyses of PSII and PSI activities were carried out in fully expanded, mature leaves of tomato plants exposed to a sublethal (0.1 mM) and a cell-death-inducing concentration (1 mM) of SA in order to reveal the differences in these parameters in the light phase of a normal photoperiod and after a prolonged dark treatment. In addition, alterations in chloroplast function and morphology were also elucidated in response to SA.

## Materials and methods

**Plant material:** Seeds of tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated at 26°C for three days in the dark; the seedlings were subsequently transferred to perlite for two weeks. Plants were grown hydroponically afterwards in a controlled environment under PPFD of 200  $\mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$  (F36W/GRO

lamps, Osram Sylvania, Danvers, MA, USA), with 12/12-h light (L)/dark (D) period, a day/night temperatures of 25/22°C, and a relative humidity of 55–60 % for eight weeks (Poór *et al.* 2011). Tomato plants ( $n = 36$ ) were treated with 0.1 mM or 1 mM SA supplied in the nutrient solution and right afterwards they were divided into two groups which received either a normal photoperiod (12-h L/12-h D, light samples) or the prolonged dark period (24-h D, dark samples) treatment for the next 24 h at 25°C. The experiments were conducted at the end of the 24-h incubation from 9 a.m. and were repeated three times. The samples were prepared from the second, fully expanded young leaves in three replicates 24 h after the different SA treatments under both conditions.

## Chl *a* fluorescence measurements and PSI activity:

Chl *a* fluorescence and P700 redox state were analysed with *Dual-PAM-100* instrument (Heinz-Walz, Effeltrich, Germany) (Klughammer and Schreiber 1994). Leaves were dark-adapted for 20 min before the measurement of  $F_0$ , the minimal fluorescence yield of the dark-adapted state, using weak measuring light when reaction centres (RC) are open. The maximal fluorescence in dark-adapted state ( $F_m$ ) was measured by applying a pulse (800 ms) of saturating light [ $12,000 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ]. The leaves were then illuminated continuously with actinic light [ $220 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ]. After 20 min, the light-adapted steady-state fluorescence ( $F_s$ ) was recorded and the maximum fluorescence level ( $F_m'$ ) in the light-adapted state was determined with saturating pulses. The actinic light was then turned off and the minimum fluorescence level in the light-adapted state ( $F_0'$ ) was determined by illuminating the leaf with 3-s far-red light [ $5 \mu\text{mol}(\text{photon}) \text{ m}^{-2} \text{ s}^{-1}$ ]. The following Chl fluorescence parameters were calculated: the maximal quantum efficiency of PSII photochemistry in the dark-adapted state:  $F_v/F_m = (F_m - F_0)/F_m$ ; the effective quantum yield of PSII:  $Y_{(II)} = (F_m' - F_s)/F_m'$ ; the quantum yield of non-regulated energy dissipation:  $Y_{(NO)} = F_s/F_m$ , and the quantum yield of regulated energy dissipation:  $Y_{(NPQ)} = 1 - Y_{(II)} - Y_{(NO)}$  (Genty *et al.* 1989, Kramer *et al.* 2004).

The quantum yield of PSI [ $Y_{(I)}$ ] is defined by the proportion of the overall P700, which is reduced at a given state and not limited by the acceptor side. It was calculated from the complementary PSI quantum yields of nonphotochemical energy dissipation:  $Y_{(I)} = 1 - Y_{(ND)} - Y_{(NA)}$ , where  $Y_{(ND)}$  and  $Y_{(NA)}$  are the quantum yields of nonphotochemical energy dissipation in PSI due to donor and acceptor side limitations, respectively.  $Y_{(ND)} = 1 - P700_{\text{red}}$  and  $Y_{(NA)} = (P_m - P_m')/P_m$ .  $P_m$  was determined by applying a saturation pulse after pre-illumination with far-red light and it represents the maximal change of the P700 signal from a fully reduced state to a fully oxidized state.  $P_m'$  was defined in the same way as the fluorescence parameter  $F_m'$ .  $P700_{\text{red}}$  was determined in a given state with the help of a saturation pulse and it represents the maximal change of the P700 signal in a given light state (Klughammer and Schreiber 1994, Schreiber and Klughammer 2008, Zhang *et al.* 2014). The yield of cyclic electron flow (CEF) around PSI [ $Y_{(CEF)}$ ] is calculated as  $Y_{(CEF)} = Y_{(I)} - Y_{(II)}$ . The ratio

of the quantum yield of CEF to the linear electron flow (LEF) is calculated as  $Y_{(CEF)/Y_{(II)}} = [Y_{(I)} - Y_{(II)}]/Y_{(II)}$  (Lei *et al.* 2014).

**Photosynthetic pigment analysis:** For pigment analysis, a two-step extraction was applied. Leaf samples of 25 mg were homogenized in 1 ml of 100% (v/v) cold acetone, and extracted for 24 h in the dark at 4°C. Samples were centrifuged ( $12,000 \times g$  for 15 min at 4°C). The pellet was extracted again with 1 ml of cold acetone/Tris buffer solution (80:20, v/v, pH = 7.8) for 24 h in the dark at 4°C. After centrifugation ( $12,000 \times g$ , 15 min, 4°C), the supernatants were collected. The pigment content was measured by spectrophotometer (Kontron, Milano, Italy) and was calculated by the equations based on Lichtenthaler and Wellburn (1983).

**Carbohydrate (soluble sugars and starch) analysis** was performed according to Hansen and Møller (1975). Briefly, soluble sugars were extracted from grinded 100 mg of fresh mass (FM) with 1 ml of 80% ethanol at 80°C for 30 min. The homogenate was centrifuged at  $2,600 \times g$  for 10 min and the supernatant was used for determination of sugar content spectrophotometrically (spectrophotometer type, producer, country) at 630 nm after reaction with anthrone using glucose (Normapur, VWR Int., Leuven, Belgium) dissolved in 80% ethanol as a standard. Then, the pellet was washed with 1 ml of deionized water, digested with 1 ml of 1.1% HCl at 100°C for 30 min, and centrifuged for 10 min at  $2,600 \times g$ . Starch concentration was determined spectrophotometrically at 630 nm with anthrone using starch (Normapur, VWR Int., Leuven, Belgium) dissolved in 1.1% HCl as a standard.

**Transmission electron microscopy:** For post-embedding electron microscopy, leaf segments (5–6 mm) were fixed in 2% paraformaldehyde and 2% glutaraldehyde solution and then further fixed for 1 h in 1% OsO<sub>4</sub>. After rinsing in buffer and dehydrating in increasing ethanol concentrations and acetone, they were embedded in Embed812 (Electron Microscopy Sciences, Hatfield, PA, USA). Embedded blocks were used to prepare semithin (0.7 µm) sections, to select the area of interest, and also ultrathin (70 nm) sections, which were mounted on nickel grids. To investigate the effects of SA treatments on the structure of chloroplasts in upper palisade mesophyll layer, three grids per block were counterstained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany) and were examined and photographed with a JEM 1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) (Talapka *et al.* 2016). Measurements were performed on digital photographs at a magnification of 20,000× with the AnalySIS 3.2 program (Soft Imaging System GmbH, Münster, Germany). Thirty experimental groups per treatments were analysed.

**Statistical analysis:** The results are expressed as means  $\pm$  SE. After analyses of variance (ANOVA) a multiple comparison followed by Duncan's multiple range test was performed with SigmaPlot version 11 software

(Systat Software Inc., San Jose, CA, USA). Means were considered significant if  $P \leq 0.05$ .

## Results

$F_v/F_m$  did not change after the treatment with 0.1 mM SA, but it decreased significantly upon 1 mM SA treatment under both light and dark treatments (Fig. 1A). There were no significant changes in the  $F_0$  and  $F_m$  parameters after SA treatments under the two environmental conditions (Fig. 1B,C). In contrast, 24 h of light deprivation caused a slight decrease in  $q_L$ , which was significant under 0.1 mM SA treatment. Interestingly, this decrease in  $q_L$  cannot be observed at the higher SA concentration (Fig. 1D).

Although  $Y_{(II)}$  was lower in control leaves kept in the dark compared to light controls, the differences between light and dark samples were not significant. However,  $Y_{(II)}$  followed decreasing trend upon increasing SA concentrations under both light and dark environment, which was significant already at 0.1 mM SA in the dark (Fig. 2A). This impact was more pronounced at 1 mM SA, where the drop in  $Y_{(II)}$  was significant compared to both controls and to 0.1 mM SA treated, illuminated plants (Fig. 2A).  $Y_{(I)}$  showed similar tendencies as  $Y_{(II)}$  during the experiments (Fig. 2B).

Although all SA treatments showed a slight increase in  $Y_{(NO)}$  compared to light-control samples, these increments were significant only upon the SA application combined with prolonged darkness (Fig. 2C).  $Y_{(NPQ)}$  also showed increasing tendencies 24 h after SA applications compared to light-control (Fig. 2E). In contrast to normal photoperiod, 0.1 mM SA caused a similar, significant increment in  $Y_{(NPQ)}$  in the dark as 1 mM SA and this increment was significant compared to both kind of control groups, but there were no significant differences between the light and dark samples in this parameter upon 1 mM SA (Fig. 2E).

$Y_{(ND)}$  followed the same trend as  $Y_{(NPQ)}$ : it increased significantly after 1 mM SA treatment in both environments, while the application of 0.1 mM SA also raised  $Y_{(ND)}$  only in the dark (Fig. 2D). In parallel,  $Y_{(NA)}$  decreased slightly during 0.1 mM SA exposure, and significantly at the higher SA concentration under both light and dark conditions (Fig. 2F). However, the drop in  $Y_{(NA)}$  caused by 1 mM SA was more significant in the samples kept under normal light conditions, than that in the dark (Fig. 2F).

Compared to the light-control,  $Y_{(CEF)}$  and  $Y_{(CEF)/Y_{(II)}}$  were enhanced by SA depending on the applied SA concentration and on the presence or absence of light (Fig. 3).  $Y_{(CEF)}$  was significantly elevated by 0.1 mM SA compared to both light and dark controls (Fig. 3A, B). However, 1 mM SA induced  $Y_{(CEF)}$  significantly only under normal light conditions. Interestingly, this means that the enhancement of  $Y_{(CEF)}$  by the dark incubation ceased in the presence of the high SA concentration (Fig. 3A). Similar tendencies can be found in case of  $Y_{(CEF)/Y_{(II)}}$  (Fig. 3B).

Nevertheless, 1 mM SA did not cause a decline in the contents of photosynthetic pigments but increased the Chl ( $a+b$ ) (Fig. 4A) and Car content (Fig. 4B) in the leaves of tomato after 24 h under normal photoperiod. At the same time, photosynthetic pigments did not change

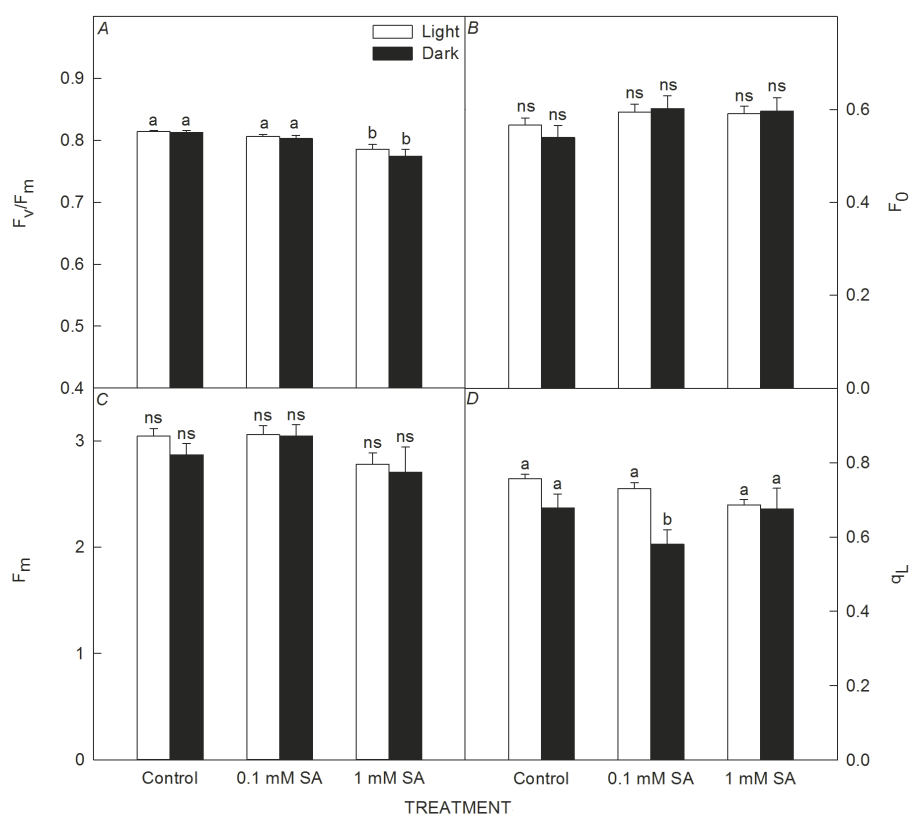


Fig. 1. Changes in the maximum quantum yield of PSII [ $F_v/F_m$ ; A], minimal fluorescence yield of the dark adapted state [ $F_0$ ; B], the maximal fluorescence in dark-adapted state [ $F_m$ ; C], and the fraction of open PSII reaction centres [ $q_L$ ; D] in fully expanded young leaves of tomato plants treated for 24 h with 0.1 or 1 mM salicylic acid (SA) in the light or in the dark. Means  $\pm$  SE,  $n = 12$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test (ns – not significant).

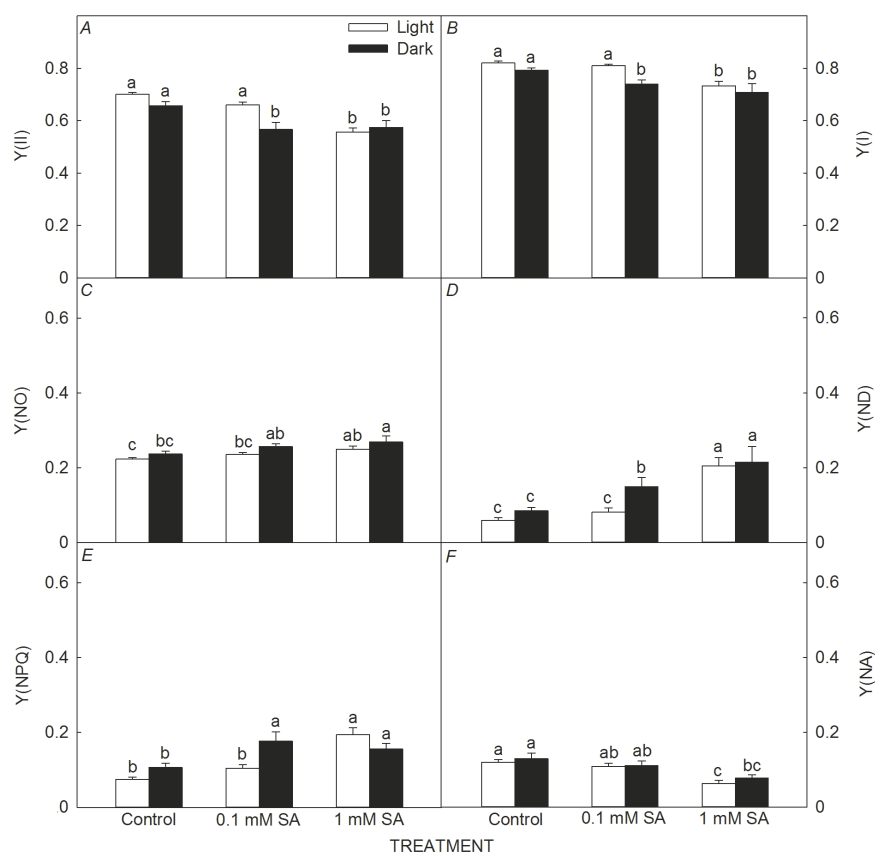


Fig. 2. Changes in the effective quantum yield of PSII [ $Y(II)$ ; A], the quantum yield of PSI [ $Y(I)$ ; B], the quantum yield of nonregulated nonphotochemical energy dissipation [ $Y(NO)$ ; C], the quantum yield of nonphotochemical energy dissipation in PSI due to donor side limitation [ $Y(ND)$ ; D], the quantum yield of regulated nonphotochemical energy dissipation [ $Y(NPQ)$ ; E], and the quantum yield of nonphotochemical energy dissipation in PSI due to acceptor side limitation [ $Y(NA)$ ; F] in fully expanded young leaves of tomato plants treated for 24 h with 0.1 or 1 mM salicylic acid (SA) in the light or in the dark. Means  $\pm$  SE,  $n = 12$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test.

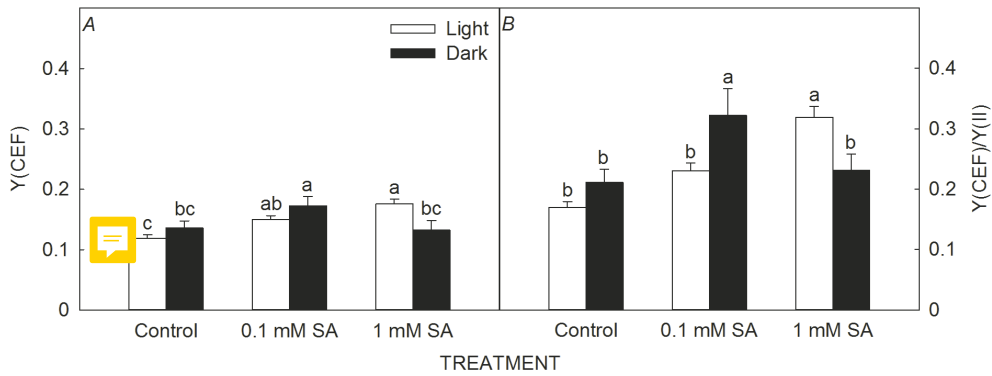


Fig. 3. Changes in the yield (Y) of cyclic electron flow (CEF) through PSI [ $Y_{(CEF)}$ ; A] and  $Y_{(CEF)}/Y_{(II)}$ ; B] in fully expanded young leaves of tomato plants treated for 24 h with 0.1 or 1 mM salicylic acid (SA) in the light or in the dark. Means  $\pm$  SE,  $n = 12$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test.

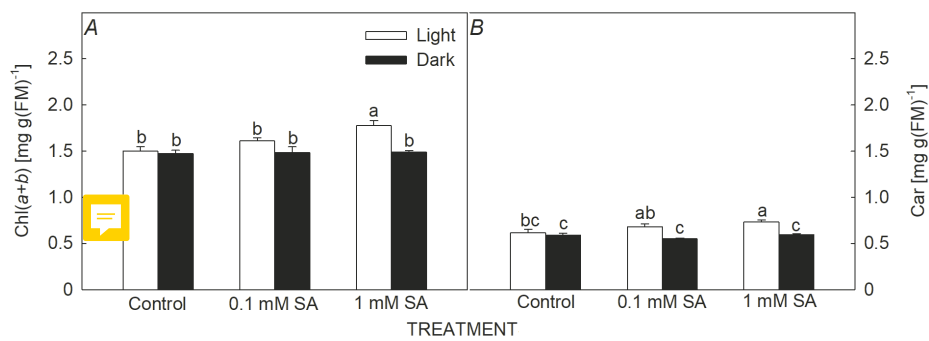


Fig. 4. Changes in the chlorophyll (a+b) [Chl (a+b); A] and carotenoids (Car) content (B) of fully expanded young leaves of tomato plants treated for 24 h with 0.1 or 1 mM salicylic acid (SA) in the light or in the dark. Means  $\pm$  SE,  $n = 6$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test.

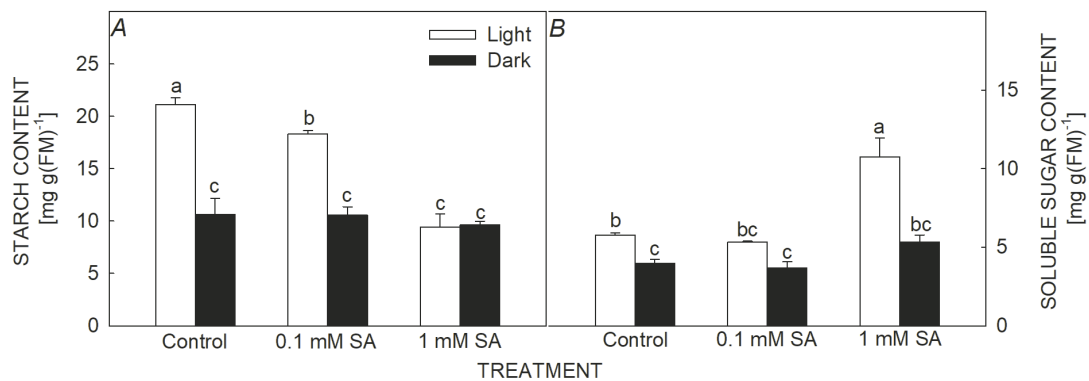


Fig. 5. Changes in the starch (A) and soluble sugars content (B) of fully expanded young leaves of tomato plants treated for 24 h with 0.1 or 1 mM salicylic acid (SA) in the light or in the dark. Means  $\pm$  SE,  $n = 6$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test.

significantly after the SA exposure in the dark (Fig. 4).

In order to detect the key products of photosynthesis after SA treatments, starch and soluble sugars contents were measured in the leaves of tomato after SA exposure. The accumulation of soluble sugars and starch was severely reduced in the leaves after 24-h dark incubation (Fig. 5). SA treatments caused a stepwise decline in starch content (Fig. 5A), and in parallel increased soluble sugar content at 1 mM SA in the leaves kept under a normal photoperiod

(Fig. 5B). However, we did not find significant differences in starch and soluble sugar contents of tissues upon SA treatments under prolonged darkness.

To detect the effects of different concentrations of SA treatments on chloroplasts structure, transmission electron micrographs (TEMs) were used. TEMs of leaf sections revealed significant changes in the fine structure of chloroplasts (Fig. 6). Control samples kept in the light or in the dark showed only small and well-known cytological



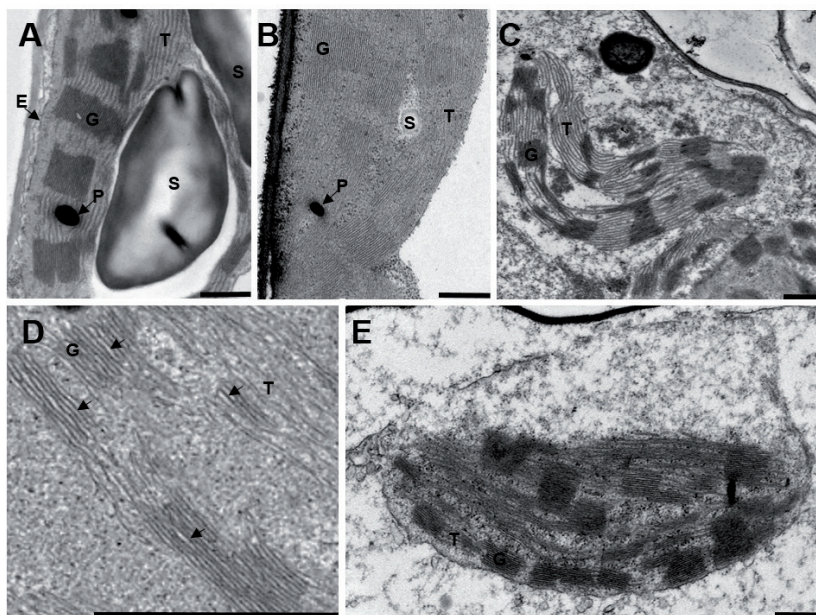


Fig. 6. Representative transmission electron micrographs (TEMs) of chloroplasts from palisade parenchyma cells of fully expanded young tomato leaves exposed to 1 mM SA for 24 h in the light or in the dark. (A) Chloroplast with normal organization and structure from a control leaf in the light. (B) Chloroplast from a control leaf in the dark. (C) Low magnification of TEMs of chloroplasts in a 1 mM SA treated leaf in the light, where some chloroplasts were damaged. (D) High magnification of TEMs of chloroplast in 1 mM SA treated leaf in the light with wavy grana and thylakoids (arrows). (E) Chloroplast in 1 mM SA treated leaf in the dark. E – chloroplast envelope, G – grana, P – plastoglobule, S – starch grain, T – thylakoids. Bar = 500 nm.

modifications in cell organelles or in chloroplast shape and structure (*e.g.* width of grana, the area of plastids) (Fig. 6A). Plastids from the control leaves in the light contained starch grains, but they were only rarely found in dark-treated samples (Fig 6B). In the leaves treated with 1 mM SA, chloroplasts lost their normal structure and exhibited spherical, longish or curved shape (Fig 6C). Moreover, dilation of thylakoid membranes, an appearance of thylakoid vesicles, and long wavy deformed lumen were also observed in these SA-treated samples (Fig 6D). In contrast, 1 mM SA induced vacuolisation of chloroplasts but not significant deformation of the lumen in the dark (Fig. 6E).

Based on the analysis of TEMs, the area of plastids was significantly higher in the light-control samples than after 24-h dark incubation (Fig. 7A). The area of chloroplasts depended on the presence of starch grains, which were observed in the samples under normal photoperiod. At the same time, SA treatments decreased the number of starch grains in these samples as a function of increasing concentration (Fig 7B). The area of plastids and the number of starch grains was very small compared to light samples and exhibited only slight changes upon SA treatments in the dark (Fig. 7A,B). Prolonged darkness increased significantly the height, but induced narrowing of grana in the chloroplasts of control plants (Fig. 7C,D). These changes in the ultrastructure were visualized in the ratio of height (H) and width (W) of grana (Fig. 7E). SA treatments decreased both parameters gradually at the applied concentrations, which were more significant in the dark-treated samples (Fig 7C,D). In contrast to the height of grana, SA application increased the height of the granal lumen, but it could be observed only in the light (Fig. 7F).

## Discussion

The presence of light is one of the most important environmental factors, which is required for optimal

growth and development of plants and can influence plant defence responses. The absence of light (*e.g.* night period or experimentally induced prolonged darkness) can alter the light-dependent signalling and regulation pathways and may induce new defence responses modulated by phytohormones (Ballaré 2014). SA is one of the key defence phytohormones and plays a crucial role in modulating immunity and disease response in plants (Loake and Grant 2007). The direct effects of SA on photosynthetic apparatus and the indirect effects on photosynthesis through the limitation of CO<sub>2</sub> supply by regulating stomatal movements take an integral part of the plant immune responses and reduce pathogen invasion by decreasing the availability of photosynthates and by inducing hypersensitive reaction (HR) in the leaves (Muthamilarasan and Prasad 2013, Cheng *et al.* 2016). Effects of SA seem to be different in light or dark conditions (Takács *et al.* 2016, Poór *et al.* 2017). Moreover, the accumulation of SA is the highest in the dark period of the day [ $\sim 0.1 \mu\text{g}(\text{total SA}) \text{ g}^{-1}(\text{FM})$  in tomato leaves]. It was found that jasmonate and salicylate accumulation patterns are regulated by the circadian clock with opposite phasing. Jasmonates peaked in the middle of the day while a peak of salicylates was found in the middle of the night (Goodspeed *et al.* 2012). In this aspect, the effects of high SA concentrations on photosynthetic apparatus and on chloroplast morphology in the dark can be especially important, because they can determine plant responses in the next light phase, especially during an infection.

It was demonstrated by our experiments that the effect of SA on chloroplast ultrastructure or on the Chl *a* fluorescence and P700 redox state parameters exhibited concentration dependency and correlated well with the availability of light. It was found by our earlier experiments (Takács *et al.* 2016, Poór *et al.* 2017) that tomato plants were able to acclimate to 0.1 mM SA, which thus can be considered as a sublethal concentration, but 1 mM SA induced a HR-like cell death program in the leaf tissues,

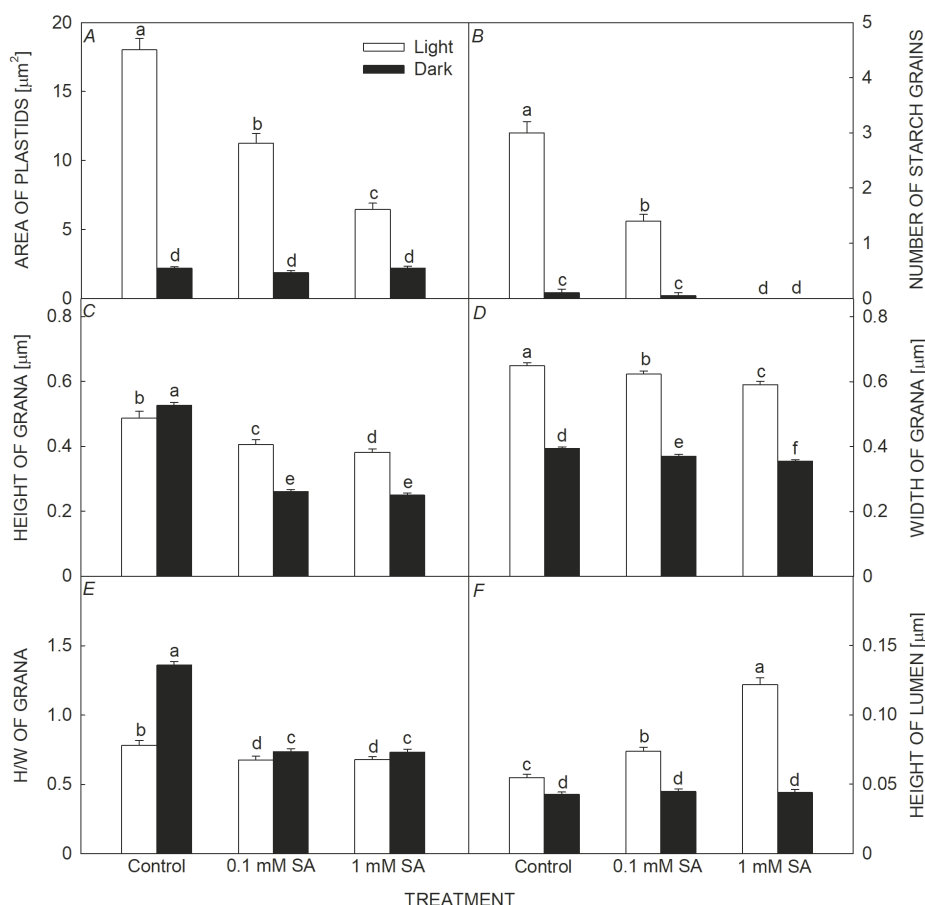


Fig. 7. Changes in the area of plastids (A), number of starch grains (B), height of grana (C), width of grana (D), ratio of height (H) and width (W) of grana (E), and height of lumen (F) in grana in chloroplasts from palisade parenchyma cells of fully expanded young tomato leaves exposed to 0.1 or 1 mM SA for 24 h in the light or in the dark. Means  $\pm$  SE,  $n = 30$ . Bars denoted by different letters are significantly different at  $P \leq 0.05$  as determined by Duncan's multiple range test.

thus it can be a model of the events associated with cell death initiation. The plants upon SA treatments exhibited much higher oxidative burst in the light than during prolonged darkness (Poór *et al.* 2017). Thus, the reactive oxygen species (ROS) generated by photosynthetic electron transport can account for SA effect in these plants. Our results demonstrated that exogenous treatment with 1 mM SA caused a significant decrease in the maximum quantum yield of PSII ( $F_v/F_m$ ) independently from the presence or absence of light during the treatment. Surprisingly, 0.1 mM SA had a significant effect on the open PSII RC populations by enhancing the closure of PSII RCs after 24-h dark incubation.

Earlier it was also found that SA decreased the  $F_v/F_m$  parameter of Chl *a* fluorescence in a concentration-dependent manner (Poór *et al.* 2011) or as a function of time (Poór and Tari 2012) and this effect of SA was dependent on increasing light intensity (Karpinski *et al.* 2013). However, this is the first observation that a 24-h dark incubation also influenced the effects of SA on the activity of photosynthetic electron transport chain. Furthermore, only a few studies have examined the effects of SA on PSI activity (Janda *et al.* 2012).

Interestingly,  $Y_{(II)}$  and  $Y_{(I)}$  were downregulated in dark-treated samples by 0.1 mM SA due to a strong CEF and therefore also enhanced  $Y_{(NPQ)}$ , although, this was accompanied with a significant increase in  $Y_{(NO)}$  as well. These changes occurred in parallel with higher  $Y_{(ND)}$ , but

$Y_{(NA)}$  was unaffected, which means that  $Y_{(I)}$  downregulation has a PSII origin. CEF can provide protection against the photoinhibition of PSII by generating a proton gradient across the thylakoid membrane through transferring electrons from PSI to plastoquinone (Takahashi *et al.* 2009). However, CEF has an important function in the prevention of PSI photoinhibition as well, by dissipating the excess electron flow to  $O_2$ , which generates superoxide anion radical.  $\cdot O_2^-$  is then utilized by the mechanism of the water-water cycle (Ort and Baker 2002). The increase in  $Y_{(NPQ)}$  is a good indicator of the onset of photoprotective mechanisms, which are mostly related to the xanthophyll cycle and the formation of  $\Delta pH$  across the thylakoid membranes. Although the yield of CEF shows a positive correlation with  $Y_{(NPQ)}$  (Miyake *et al.* 2010, Lei *et al.* 2014, Zhang *et al.* 2014), the formation of  $\Delta pH$  across the thylakoid membranes and therefore the increase in  $Y_{(NPQ)}$  could be induced partially by CEF-independent mechanisms (e.g. NAD(P)H-oxidoreductase dependent chlororespiration) at high SA concentrations during darkness (Miyake *et al.* 2010), or by other,  $\Delta pH$ -independent processes (Jahns and Holzwarth 2012). At the same time, 0.1 mM SA caused only very small changes in  $Y_{(II)}$  and  $Y_{(I)}$ ,  $Y_{(NPQ)}$ , and  $Y_{(ND)}$  in the light-sample.

$Y_{(II)}$  and  $Y_{(I)}$  were also downregulated by 1 mM SA, but the decreases suggest different underlying processes in dark and light samples. In light samples, high CEF-induced NPQ protected the photosystems more efficiently.

In contrast to this, in dark samples, CEF was slightly less efficient, and therefore  $Y_{(NO)}$  significantly increased. These observations in parallel with a slight increase in  $F_0$  can suggest that the PSII super-complex might be damaged. However, NPQ mechanisms can dissipate the excess energy absorbed by LHCII and in cooperation with the antioxidant system, it can alleviate the harmful effects of ROS on photosynthetic apparatus (Müller *et al.* 2001). Based on our results, SA treatments stimulated both  $Y_{(CEF)}$  and  $Y_{(NPQ)}$ , especially in the case of 0.1 mM SA in the dark, suggesting that there is a greater need for photoprotective mechanisms after the dark incubation. Night effect on the Chl fluorescence parameters of Norway spruce seedlings (adapted to normal photoperiod) was also investigated earlier (Roháček and Barták 1999). The authors found that after 6 h of darkness quenching analysis of Chl *a* fluorescence showed an increased NPQ associated with decreased  $q_P$  and  $Y_{(II)}$ . SA-induced damage of the photosynthetic apparatus was also alleviated by enhanced photoprotection, which was more effective at the lower SA concentration in the dark. Nevertheless, under these conditions of SA exposure, contents of Chl (*a+b*) and Car did not decrease, which could have modified the Chl *a* fluorescence parameters.

The accumulation of the photosynthetic products was also different after SA exposure in the two environments. Prolonged darkness significantly decreased starch and soluble sugar contents in the control leaves, but it was not affected further by SA. However, SA caused significant changes in carbohydrate contents in the light. SA of 1 mM decreased starch but increased the soluble sugar content of these samples. High contents of sucrose and glucose can trigger leaf senescence (van Doorn 2008), thus the absence of starch synthesis and a concomitant accumulation of soluble sugars may contribute to the initiation of cell death by high concentrations of SA.

Chloroplast morphology was also altered after SA accumulation in barley plants (Uzunova and Popova 2000). Our results demonstrated that the effects of SA on the chloroplast structure were also dependent on the presence or absence of light. SA treatments, as a function of the applied concentration, decreased the area of chloroplasts in the light but this parameter remained constant and independent of SA under prolonged darkness. Shape and area of chloroplasts can be determined by the presence and quantity of starch grains, which were also regulated by the diurnal cycle (Gebauer *et al.* 2017, O'Leary *et al.* 2017). Interestingly, the area of chloroplasts in parallel with the number of starch grains decreased more effectively in the light, but the height of grana decreased more pronouncedly after SA exposure in the dark compared to the light. Moreover, we could observe long, wavy, deformed lumen and dilation of thylakoid membranes after 1 mM SA treatment in the light. The structural stability and optimal size of grana are dependent on the formation of PSII-LHCII super-complex (Kirchhoff 2014). Thus, changes in the PSII-LHCII super-complex, such as the decrease in the number of LHCII (Kim *et al.* 2009) and/or decrease in the number of PSII (Iwai *et al.* 2014), affect the grana formation and decrease the grana

size. Moreover, photosynthetic membranes are rich in the non-phosphorous and uncharged glyceroglycolipids. The two main types, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) play also a role in the stabilization of photosystem protein complexes in chloroplasts (Rocha *et al.* 2018). It has been observed recently that 1 mM SA changed the MGDG/DGDG ratio and decreased the MGDG content in flax leaves (*Linum usitatissimum* L.), which can contribute to the changes in biogenesis and stabilization of chloroplast membranes (Belkadi *et al.* 2015). The decrease in  $F_v/F_m$  and  $Y_{(II)}$  upon 1 mM SA treatment accompanied with a slight increase in  $F_0$  under both light and dark conditions suggests that the PSII super-complex may have been damaged and/or the turnover of D1 may have been disturbed. SA plays an important role in regulating the thylakoid membrane proteins under different environmental stresses. Interestingly, Chen *et al.* (2016) found that 0.25 mM SA increased the content of D1, Lhcb3, and Lhcb6 but did not change the content of Lhcb1, Lhcb2, Lhcb4, and Lhcb5 in two-week-old wheat seedlings after 24 h. In addition, 0.3 mM SA was able to influence the turnover of D1 protein through regulating the activity of protein kinases in wheat leaves (Zhao *et al.* 2011). In contrast, Gao *et al.* (2018) observed that D1 and the major PSII and PSI proteins significantly decreased in the presence of 600  $\mu$ M SA in *Chlamydomonas reinhardtii* and SA caused the digestion of thylakoid membrane proteins. Moreover, D1 subunit is the main target of photooxidative damage (Kirchhoff 2014), which can be induced by SA in the chloroplasts (Poór and Tari 2012). Thus the potential decrease in D1 content can contribute to the decrease in grana height, which was found also in the case of light stress (Herbstová *et al.* 2012). Kirchhoff *et al.* (2011) observed in dark- and light-adapted *Arabidopsis* leaves that the granal thylakoid lumen significantly expanded and swelled in the light, which was prevented by a restriction imposed on protein diffusion in the dark. The swelling of the lumen could allow luminal Deg proteases to get in contact with PSII contributing to the repair of photodamaged PSII proteins (Kirchhoff 2014). In addition, Mishev *et al.* (2011) found that the expression of the genes for the two plastid proteases Deg1 and FtsH5 involved in D1 protein degradation was inhibited in leaves of *Arabidopsis* in the dark, suggesting that these proteases function mainly in illuminated leaves. These results may suggest that PSII reaction centres need higher repair capacity in the light after 1 mM SA treatment than in the dark.

In conclusion, the effects of exogenous SA application on photosynthetic activity and on chloroplast morphology are different under normal photoperiod and after 24-h incubation in prolonged darkness. Although the treatment of tomato plants with the sublethal concentration of SA decreased the effective quantum yield in both photosystems after dark incubation, the photoprotective processes, such as the regulated and nonregulated nonphotochemical quenching in parallel with CEF were activated effectively in the mature leaves of tomato. With the exception of CEF, low SA did not cause significant changes in these parameters under normal photoperiod. However, the low



SA concentration exerted an influence on chloroplast morphology, such as the area of plastids, the height of grana or granal lumen, under normal photoperiod and/or during prolonged darkness. In contrast to the lower concentration, 1 mM SA elicited an inhibition in  $F_v/F_m$ ,  $Y_{(II)}$ ,  $Y_{(I)}$  in both environments, which was accompanied with less efficient photoprotection especially after 24-h dark treatment. SA of 1 mM caused damaged chloroplast structure (abnormal shape of plastids, increased volume of grana thylakoids) but it was more pronounced in the light. In addition, chloroplasts lost their normal thylakoid arrangement and displayed damaged envelopes. Decrease in grana height, as well as dilation of thylakoids and appearance of long wavy membranes with deformed lumen were also observed in these SA-treated samples in the light which is in good agreement with our earlier finding that 1 mM concentration of SA induced cell death in tomato leaves, which can be more considerable in the light.

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